Expert Opinion

Differential receptor-mediated drug targeting to the diseased brain

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The brain is not directly accessible for intravenously administered macro- and most small molecular drugs because of the presence of the blood–brain barrier (BBB). In this respect the BBB functions as a physical and metabolic barrier which is presented by the endothelial cells in brain capillaries. In order to overcome the BBB, therapeutic compounds have been targeted to internalizing receptors at the BBB. In this review we summarize the different approaches that have been described in current literature, including the possible difficulties for clinical application. Particularly, we focus on the possible impact of brain diseases on receptor-mediated transport to the BBB/brain and how this may affect various targeting strategies. Moreover, it is our opinion that a differential drug targeting/delivery approach should be applied to treat central nervous system (CNS) diseases that are related to the BBB alone, and for CNS diseases that are related to both the brain and the BBB.

Keywords: blood–brain barrier, disease-induced targeting, protein and gene delivery, receptor-mediated targeting

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1. Barriers in the brain

Three barriers limit drug transport to the brain parenchyma. These include the blood–brain barrier (BBB) localized in the capillaries of the brain; the blood–cerebrospinal fluid barrier (BCSFB), which is presented by the choroid plexus (CP) epithelium in the ventricles; and the ependyma, which is an epithelial layer of cells covering the brain tissue in the ventricles and limiting the transport of compounds from the cerebral spinal fluid (CSF) to the brain [1]. The epithelium of the BCSFB forms a major barrier to the transport of drugs into the ventricle. Moreover, once a drug has entered the ventricle it can poorly enter the brain parenchyma because of the presence of the ependyma. In this review drug transport to the BBB/brain is discussed. Since the surface area of the BBB is facing the blood and that of the BCSFB is facing the ventricle, the BBB is considered to be the most important barrier for drugs entering the brain from the blood side. Ehrlich [2] was the first to show evidence for a barrier between the bloodstream and the brain. He injected vital dyes intravenously and found that, in contrast to other tissues, they did not stain the brain. In contrast, Goldman [3] injected these dyes into the CSF, after which staining of the brain, but not of the peripheral organs, was observed. This has lead to the concept of the BBB, which has been demonstrated histologically by electron-microscopy as well. The human BBB has a total blood vessel length of approximately 600 km. In fact, every cm^3 of cortex comprises the amazing sum of 1 km of blood vessel, causing almost every neuron to have its own capillary. The BBB is mainly formed by brain capillary endothelial cells (BCEC), although other cell types such as pericytes, astrocytes and

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neuronal cells also play an important role in the function of the BBB. BCEC have specific characteristics, such as tight junctions, which prevent paracellular transport of small and large (water soluble) compounds from the circulation to the brain. Furthermore, transcellular transport from blood to brain is limited as a result of low vesicular transport, high metabolic activity and a lack of fenestrae. The BBB functions as a physical, a metabolic and an immunological barrier. These specific characteristics of the BBB are induced and maintained by the (endfeet of) astrocytes surrounding the BCEC, as well as by neuronal endings, which can directly innervate the BCEC. Pericytes also play a role at the BBB, as they share the continuous capillary basement membrane with the BCEC. Their phagocytotic and homeostatic activity forms an additional BBB property [1].

1.1 BBB protective mechanisms

In the context of drug transport to the brain, the BBB is not only a physical but also a metabolic barrier. Moreover, because of the presence of the BBB, the brain can be considered as a sanctuary site, making it difficult for many (macro- and small molecular) drugs to actually reach the brain. Paracellular transport of hydrophilic compounds across an intact BBB is virtually absent due to the presence of narrow tight junctions. Transcellular transport to the BBB/brain occurs only by diffusion of lipophilic compounds, adsorptive mediated endocytosis (pinocytosis) and by influx (and efflux) transporters at the BCEC's presenting the BBB [4,5].

In this review we will focus on receptor mediated endo- and transcytosis as an approach for targeted delivery of large molecular compounds (e.g., proteins, plasmids, RNAi), since these systems are particularly well-suited to transport such compounds to the BBB/brain. However, the contribution of other transport systems (e.g., adsorptive mediated endo/transcytosis) should not be neglected since they may play an important role in the efficacy of treatments, especially under inflammatory disease conditions (discussed in paragraph 2).

1.2 BBB intracellular protective mechanisms

Not only the tight junctions and transporters present on BCECs, but also the intracellular metabolic activity of these cells presents an important part of the barrier to drug delivery. Following endocytosis into BCECs, compounds can follow various intracellular routes that may influence their availability in the BBB and the brain. Upon receptor-mediated internalization, clathrin and non-clathrin-coated vesicles are formed, which are approximately 120 nm in diameter [6]. These vesicles may transport their contents to the other side of the cell followed by exocytosis or go into a route leading to degradation. Indeed at least two important routes for degrading proteins have been identified including the lysosomal and the ubiquitin–proteasome route [7]. For DNA and RNA there may be also endosomal and/or lysosomal breakdown. DNAses, RNAses (including the RISC complex and endo- and exonucleases) may play an important role in the breakdown of these molecules. Clearly, these processes will lead to a reduced intracellular availability of proteins, (si)RNA and genes and consequently to a diminished transport further into the brain.

Therefore, in targeting drugs to the BBB/brain, one should particularly focus on receptor-mediated transport systems that do not follow the degradation pathways following internalization, or that can escape from endosomal or lysosomal degradation.

1.3 Receptor-mediated brain targeting

The poor transport of drugs to the BBB/brain is a serious problem in the treatment of many central nervous system (CNS) diseases such as Alzheimer's disease, Parkinson's disease, stroke, depression, epilepsy, migraine and other brain diseases. To solve this problem several methods have been described that can be subdivided into different approaches:

- local delivery by direct injection;
- induction of enhanced BBB permeability;
- intranasal administration [8]; and
- systemic physiological BBB targeting strategies.

In this review we will focus on global physiological BBB targeting strategies of macromolecules. These strategies involve the application of a homing device that utilizes endogenous transport mechanism(s) for site-specific delivery in the brain. The advantage of active targeting is the potential increase of the amount of delivered drug in the target tissue, thereby increasing its pharmacological response. In addition, such an increased therapeutic efficacy, due to a lower dosage of the drug, may reduce systemic side effects. However, these side effects depend very much on the particular macromolecule applied, the distribution of the receptors in the body and the intracellular fate of the drugs in the various cell types, which makes this an important issue in receptor-mediated targeting approaches. Moreover, targeting efficiency to the brain can be enhanced by using inducible receptors that are known to be upregulated under inflammatory CNS disease conditions. An example of such an inducible receptor is the diphtheria toxin receptor, which is discussed in more detail in this section. Furthermore, various devices can be used to target macromolecular drugs to the brain such as conjugates, liposomes, polymer systems or solid nanoparticles.

In the next section several examples are summarized of receptor-mediated transcytosis that have been applied to target drugs to the BBB/brain (summarized in **Table 1**).

1.3.1 Transferrin receptor

The most widely used and best characterized system for receptor-mediated drug targeting is the transferrin receptor (TfR). The TfR mediates uptake of iron bound to transferrin in hepatocytes, erythrocytes, intestinal cells and monocytes. Furthermore the TfR is present on endothelial cells of the

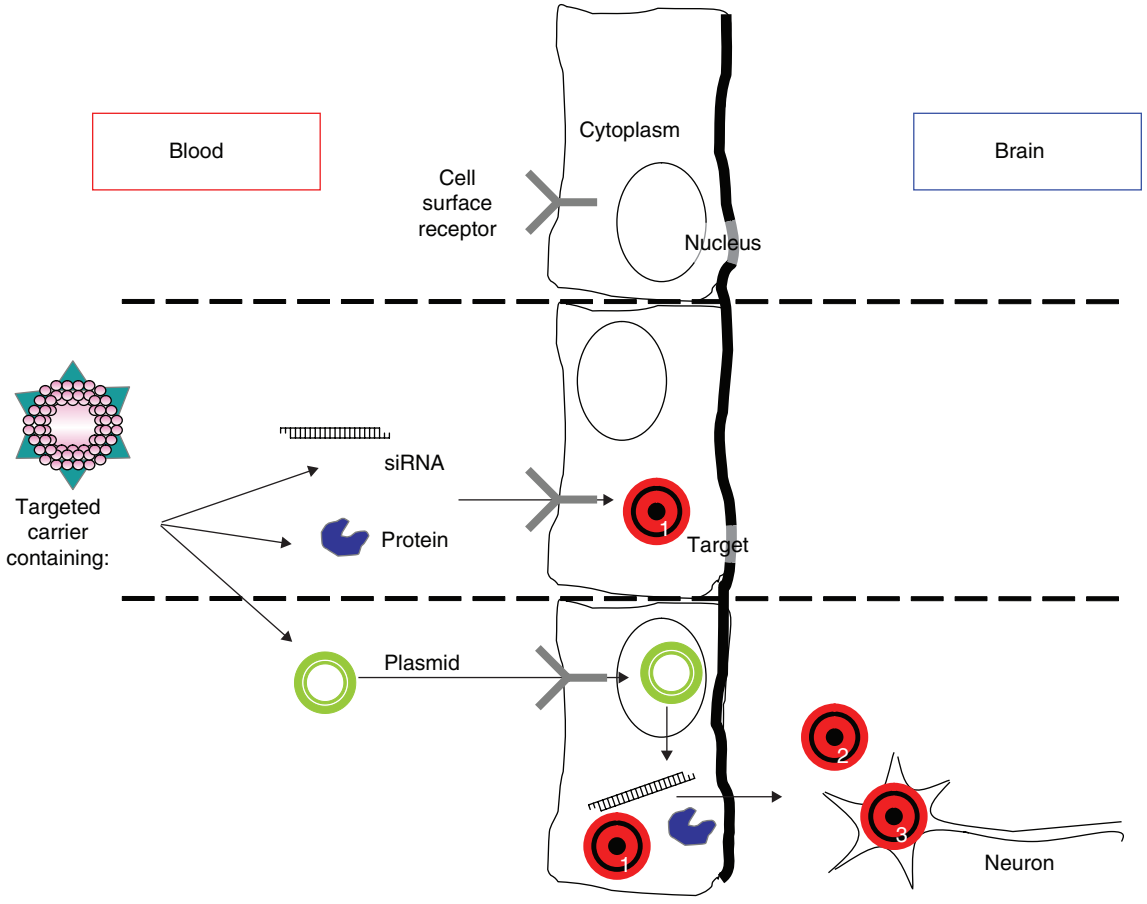

Figure 1. Differential macromolecular drug targeting to the BBB/brain. 1. Intra-cellular targets in the brain capillary endothelial cells. 2. Extra-cellular targets in the brain's extra-cellular space and/or neuronal/glial cell surface. 3. Intracellular targets in brain cells, including neurons, astrocytes, dendritic cells and pericytes.

BBB and on choroid plexus epithelial cells and neurons. Various methods for targeting drugs to the TfR have been described. Antibodies directed against this receptor (OX-26), the endogenous TfR ligand transferrin and TfR binding peptides can be used as carrier molecules to target drugs to the TfR. These well-described strategies have their advantages and disadvantages, as reviewed previously [9-14]. They have been shown to be effective *in vitro* and in animal studies treating several brain diseases with large molecular drugs combined with targeting devices like protein conjugates and liposomes [15-17]. Nevertheless, the potency of receptor-mediated targeting to the brain is impressive, despite the difficulties in human application of targeting compounds to the TfR.

1.3.2 *Insulin receptor*

Pardridge *et al.* have extensively researched the insulin receptor for BBB targeting. This method has been well characterized and reviewed [10-12]. Targeting the insulin receptor using insulin itself is troublesome since it interferes with endogenous insulin metabolism and consequently high dosages are lethal. Therefore, targeting to the insulin receptor has been performed using monoclonal antibodies (MAb) that bind to an exofacial epitope of the insulin receptor. Upon binding the receptor-MAb complex is internalized. Murine antibodies against the human insulin receptor have been used in primates to diagnose Alzheimer's disease using an amyloid-B peptide and have furthermore been used to target plasmid DNA to the brain. A humanized antibody has been developed for human application and was tested in several primate studies demonstrating the presence of radioactively labeled therapeutic proteins in the brain. The same targeting device was used to target genes to the brain using Trojan horse liposomes. Transgene expression has been observed following delivery to brain tissue both in diseased and healthy brains. However, no therapeutic effects following systemic administration of these complexes in disease models have been shown so far.

1.3.3 *Lipoprotein receptors*

Lipoprotein receptor-related protein (LRP)1 and LRP2 are related to the cell surface Low-Density Lipoprotein Receptor (LDLr). All these receptors are expressed on the BBB and

Table 1. Receptor-mediated targeting strategies that have been used for the delivery of drugs to the BBB/brain.

	Target receptor/transporter	Substrates	Conditions	Receptor fate	Ref.
1	Transferrin receptor (TF_R)	Fe-Transferrin, Mab	AD	Expression down	[9-14]
2	Insulin receptor (INS_R)	Insulin, HIRMab	AD	Sensitivity decreased	[10-12]
3	Lipoprotein receptor-related protein (LRP1 and LRP2)	Lactoferrin, Angiopep® melanotransferrin, ApoE	AD	Expression up	[20-22,24,25,89-91]
4	Scavenger receptor class B, type I (SR-B1)	ApoA1	Unknown	Unknown	[27]
5	α(2,3)-sialoglycoprotein/ TMEM30A	FC5 (single domain llama Mab)	Unknown	Unknown	[28]
6	Acetylcholine receptor (AchR)	Rabies virus glycoprotein peptide	AD, PD, epilepsy	Expression down	[30]
7	Diphtheria toxin receptor (DT_R)	Diphtheria toxin, CRM197	AD, PD, MS, epilepsy, ischemia, glioma	Expression up	[38,39,92]
8	Glutathione transporter	Glutathione	AD, PD, Ischemia	Unknown	[41,88,93]

AD: Alzheimer's disease; PD: Parkinson's disease.
Examples of receptor regulations during disease conditions are indicated: for more details see paragraph 2.2.2.

are multifunctional, multi-ligand scavenger and signaling receptors [18]. The importance of LRP function in the CNS is emphasized by its high expression in the cerebellum, cortex, hippocampus and brain stem [19]. Several substrates for this receptor family have been characterized and some of them have been used for brain targeting strategies.

Firstly, transport of lactoferrin across the BBB has been documented. Lactoferrin is an iron (Fe^{3+})-containing protein with antimicrobial activity. Transcytosis of lactoferrin is LRP-dependent, as shown in an *in vitro* BBB model [20]. Furthermore, *in vivo* studies show uptake of lactoferrin in the brain [21]. Secondly, melanotransferrin can be used for receptor-mediated transcytosis across the BBB via the LRP1 receptor [22]. Melanotransferrin is a membrane-bound transferrin homologue that also exists as a soluble compound. Intravenous application of melanotransferrin results in uptake of a small proportion to the brain and delivery of the majority of the injected dose to the kidney and the liver [23]. Finally, more recent publications describe the selection of Kunitz domain-derived peptides for BBB penetration. One of these peptides (called Angiopep-2®, AngioChem Inc., Canada) is an effective vector for brain delivery. Furthermore, LRP1 has been shown to be involved in the transcytosis process [24] and conjugates of anti-cancer compounds and angiopep-2 have shown to be active against brain tumors in animals [25].

1.3.4 *Scavenger receptor class B type I*

Apolipoprotein A1 (ApoA1) is the major apolipoprotein component of high density lipoproteins (HDL). Cholesteryl ester and vitamin E from HDL are taken up by cells upon binding of ApoA1 to scavenger receptor class B type I (SR-B1). This SR-B1 is a multiple ligand receptor but it has a particularly high affinity for ApoA1. SR-B1 is furthermore expressed on BCEC [26]. *In vitro* BBB transport of nanoparticles coated with ApoA1 has been shown [27]. Thus using ApoA1 as a carrier molecule to target the BBB and brain seems feasible.

1.3.5 *TMEM30A*

A llama single-domain antibody phage-display library has been used to select antibodies that recognize human cerebromicrovascular endothelial cells. The selected antibodies have been tested for BBB transport both *in vitro* and *in vivo*. It was shown that the transport pathway of the selected antibody (designated FC5) involved clathrin-coated endocytosis, suggesting a receptor-mediated uptake mechanism and subsequently transcytosis at the BBB. The responsible receptor was suggested to be α(2,3)-sialoglycoprotein [28]. Further research has identified a novel receptor that is capable of binding FC5 and is present at the BBB, which has been termed TMEM30A. The function of this molecule is unknown but it belongs to the P4-ATPase family [29].

1.3.6 *Acetylcholine receptor*

A recent paper showed the application of a peptide derived from rabies virus glycoprotein to target siRNA to the acetylcholine receptor on endothelial cells. *In vitro* studies have provided evidence of uptake and binding of the peptide siRNA complex in neuronal cells, while uptake in brain capillary endothelial cells was not observed. *In vivo* experiments demonstrated therapeutic effects on viral encephalitis and the presence of siRNA in the brain has been confirmed [30]. However, as the authors say, much has to be done to optimize these systems.

1.3.7 *Diphtheria toxin receptor*

Diphtheria toxin is taken up by cells via receptor-mediated endocytosis. It uses the membrane-bound precursor of heparin-binding epidermal growth factor (HB-EGF) as its transport receptor [31]. This proHB-EGF is also known as the diphtheria toxin receptor (DT_R). The DT_R is expressed on the BBB, neurons and glial cells [32]. Upregulation of DT_R expression has been observed during and following inflammatory conditions induced by several diseases. A brain-specific upregulation has also been shown in diseases like tumors, epilepsy and stroke [33-35]. This most likely occurs during other diseases in the brain in which inflammatory processes play a crucial role, like Alzheimer's disease, Parkinson's disease, multiple sclerosis, encephalitis, lysosomal storage diseases, etc. This suggests that local upregulation of the DT_R, as a consequence of inflammatory conditions during brain diseases, can be used for disease-induced specific targeting. Another interesting aspect of diphtheria toxin is its intrinsic endosomal escape mechanism which allows the catalytic domain of the protein to enter the cytosol of the target cell, thereby bypassing the lysosomal degradation system [31]. This provides opportunities for targeted delivery of drugs to the cytosol of brain capillary endothelial cells. DT is not suitable for application as a carrier molecule for targeting the DT_R because of its toxicity. However, a non-toxic mutant form of DT, called CRM197, is available as a human applicable carrier protein for targeted delivery to the brain. CRM197 is safe since it is already in use in human vaccines and also as a therapeutic protein to scavenge the soluble form of HB-EGF, thereby inhibiting the growth of tumors [36,37]. Recent publications show that CRM197 can carry molecules across the BBB both *in vitro* and *in vivo* [38,39].

1.3.8 *Glutathione transporter*

Kannan *et al*. have shown the presence of a transporter for glutathione at the BBB [40]. In addition, glutathione-mediated delivery has recently been filed as an approach to target drugs to the brain [41]. Furthermore, this transport mechanism was proposed as a target for the treatment of Parkinson's disease. [42]. Glutathione is endogenously expressed, has favorable antioxidant-like properties and plays a central role in the detoxification of intracellular metabolites. Its influx by the glutathione transporter may also be mediated by sodium coupled transporters, the organic anion transporters (OAT1 and OAT3) and the sodium-dicarboxylate 2 exchanger (SDCT2). Whether the glutathione transporter is an internalizing receptor or a membrane carrier is still unknown. Importantly, energy-dependent glutathione efflux transporters, like the multi-drug resistance proteins (MRP) and the organic anion transporting polypeptide 1 (OATP1), are capable of eliminating glutathione from target cells. This may counteract the targeted delivery of drugs to the BBB/brain, which should be taken into account when applying glutathione as a carrier molecule. However, since glutathione transporters are highly conserved across all mammalian species, performing animal experiments seems a promising approach to validate the potential uses of glutathione as a targeting strategy.

2. BBB/brain targeting and the influence of disease status

BBB protective mechanisms are often diminished in diseases that affect the brain. In addition, most brain diseases change the BBB integrity. This has been described for Alzheimer's, Parkinson's, epilepsy, ischemia, HIV and multiple sclerosis, amongst other brain diseases [43-46]. The inflammatory response that is associated with these diseases is a process that is common to all of the above-mentioned brain diseases and is of crucial importance to the integrity of the BBB [47-49]. In addition, it is well known that BBB properties are also influenced by drugs such as glucocorticoids and interferons. This will influence the functionality and thus the permeability of the BBB during such conditions. In addition, drug delivery to the brain will also be altered [48,50,51]. Moreover, the disposition of drugs in the brain (neuro-pharmacokinetics) during diseases may influence the treatment efficiency of brain diseases [52].

Changes in BBB permeability by CNS diseases may be mediated through changes in specific transport or, alternatively, by effects on non-specific transport pathways. This holds for both paracellular transport (tight-junction mediated) and transcellular transport routes like adsorptive mediated endocytosis (pinocytosis) or receptor-mediated endocytosis. Subsequently this potentially increases the efficacy of drugs in the brain. Thus, drugs that normally are unable to cross the BBB may reach their target areas in the diseased brain due to this enhanced permeability and/or retention.

2.1 Paracellular transport in disease state

Tight junctions (TJs) are essential for the regulation of brain homeostasis and protect the microenvironment of the brain. In many brain pathologies TJs are disrupted, resulting in an increased paracellular transport. Inflammatory conditions present in most brain diseases lead to the production of cytokines that result in TJ disruption. This has been clearly shown in many *in vitro*, animal and human studies. These effects have been shown to be mediated via the expression of proteins essential for TJ integrity. One of the main causes is the formation of oxidative stress under inflammatory disease conditions (e.g., the formation of radical oxygen and nitrogen species), resulting in downregulation of occludin expression [48,53]. Occludin, ZO-1 and ZO-2 play a pivotal role in TJ formation, as recently reviewed [54]. A secondary cause leading to TJ disruption is due to a rise in intracellular free calcium levels caused by inflammatory mediators [55].

By using *in vitro* models with brain capillary endothelial cells, it has been shown that an increase in cell layer permeability can be induced by cytokines that are released

during inflammatory conditions, like TNF-α, IFN-γ, IL6, IL17 and IL22 [56-60]. Other studies show a direct effect of lipopolysaccharide (LPS), which has commonly been used to induce meningitis-like inflammation in BBB models [61,62], on BBB integrity. Similarly, stroke, which is characterized by deprivation of blood, leading to ischemic conditions, followed by reperfusion of an area of the brain, is associated with TJ disruption. Indeed, hypoxic conditions have been used *in vitro* to mimic ischemia in the brain, resulting in an increased permeability by alterations in TJ protein expression [63,64]. Moreover, direct injection of LPS or TNF-α in the CNS increases BBB permeability *in vivo* [65,66]. Similar effects have been observed in other animal models with various brain diseases. In a mouse model for Alzheimer's disease, increased BBB permeability has been found, leading to increased transport of antibodies against amyloid-β to the brain.[67] A similar effect was observed using ischemia models where upregulation of cytokines like TNF-α and IL-1 was shown [68,69]. Breakdown of TJs has been observed in infectious diseases caused by viruses or bacteria, resulting in an increased BBB permeability. HIV, meningitis and encephalitis are a few examples of infectious diseases that increase BBB permeability [70,71]. Even peripheral inflammatory diseases can disrupt BBB integrity due to an increased level of circulating cytokines. This has been demonstrated in animal models with visceral inflammation [72,73].

Interestingly, in humans increased cytokine levels coincide with a high BBB permeability in MS. Moreover, TJ disruption has been found in brain tissue from MS patients [74,75]. In HIV patients, activated microglia have been shown to release cytokines that disrupt the BBB, eventually resulting in neurodegeneration [76]. Examples such as these make it tempting to speculate that the increased paracellular transport as a consequence of TJ disruption during diseases opens up possibilities for better drug delivery to the brain.

2.2 Transcellular transport in disease state

2.2.1 *Adsorptive mediated endocytosis (pinocytosis)*

Cationic (targeting) molecules enter the brain via adsorptive mediated endocytosis [77,78]. Changes in endocytotic activity of the endothelial cells in disease state do not only affect this transport route, but may also influence the efficacy of other strategies like receptor-mediated endocytosis. Studies in CNS endothelial cells *in vitro* have shown an increased adsorptive mediated endocytosis after TNF-α or IL-6 treatment [79]. In addition, increased endocytotic activity has been observed in rats with an occluded middle cerebral artery as a model for brain ischemia [80]. Whether these endocytotic processes are elevated in humans during disease conditions is incompletely understood, but given the generalized inflammatory response that often accompanies these CNS diseases, such increased endocytotic activity seems highly likely.

2.2.2 *Receptor-mediated endocytosis*

Macromolecules that are taken up in the brain via internalizing receptors present on the luminal side of the brain endothelial cells can be used as carrier proteins to target drugs to the brain (see paragraph 1.2). The expression of their target receptors is often influenced by diseases and the progression of the disease treatment. Whether this is favorable or unfavorable for drug targeting largely depends on the type of receptor, the regulation of its expression and its distribution in the brain. Thus, importantly, when applying such receptors in targeting strategies as mentioned in paragraph 1.2, targeting efficiency may be changed during disease state. This should be taken into account when choosing a targeting approach. Here we will focus on the Tf-receptor, insulin receptor, LRP-receptor, acetylcholine receptor, DT-receptor and the glutathione transporter.

1. The TfR mediates the uptake of iron bound to transferrin in the brain and the BBB. A decrease in TfR expression in the hippocampus has been shown in Alzheimer's disease. Although TfR presence is not studied in all brain diseases, it is clear that the availability of iron in the brain plays a pivotal role in the progression of brain diseases [81]. Therefore, iron metabolism may influence the efficacy and safety of TfR-mediated targeting to the brain.
2. The insulin receptor has an important function in diabetes and obesity. Sensitivity of the insulin receptor is altered during these diseases and also during age-related brain diseases like Alzheimer's disease [82]. Targeting the insulin receptor in an attempt to treat a certain disease may thus result in a disturbance of insulin metabolism, insulin resistance or may influence the efficacy of drugs targeted to this receptor, thereby causing unwanted side effects.
3. As previously described, the LRP receptor is highly expressed in the cerebellum, cortex, hippocampus and brain stem [1]. Elevated expression of LRP protein on capillary endothelial cells potentially indicates a dysfunction of the BBB and the regulation of beta-amyloid transport in Alzheimer's disease. The importance of this receptor in brain development and tissue repair has furthermore been shown [83]. Although the role and regulation of the LRP receptor is not completely understood, it is very likely that expression of the receptor is affected by most brain diseases that have an inflammatory component. Several studies have shown inhibition of inflammatory processes by LRP [84]. The effects of brain diseases on SR-B1, α(2,3)-sialoglycoprotein or the TMEM30A are not known.
4. The acetylcholine receptor plays a pivotal role in the cholinergic system and its expression is clearly affected by brain diseases. Reduced expression of the acetylcholine receptor has been found in patients with Alzheimer's disease and an important role of the receptor has been described in Parkinson's disease and epilepsy [85]. Moreover, this receptor is also a target in the treatment of these brain diseases using nicotine.

5. The expression of the DT_R is strongly upregulated under inflammatory disease conditions. These inflammatory conditions occur in many brain diseases, for example Alzheimer's disease, Parkinson's disease, multiple sclerosis and ischemia. Indeed, it has been shown that the expression of DT_R is increased under disease conditions like stroke and gliomas [34,86]. In addition, upregulation of DT_R mRNA in rat brain has been shown following epileptic seizures [35]. Consequently, this receptor upregulation in the diseased area may enhance the therapeutic efficacy and result in disease-induced targeting. Here the elevated paracellular transport that is often associated with the above-mentioned disease conditions, may also contribute to the delivery and targeting to the DT_R on neurons and glial cells. However, two possibly complicating properties of DT_R expression and functioning are the necessity of the presence of co-factors for drug–CRM197–DT_R complex internalization and the protease-mediated shedding of the membrane-associated DT_R, which may result in reduced availability of the receptor for drug targeting.
6. Since glutathione acts as an antioxidant, it plays a major role in brain diseases. Due to the anti-oxidative properties of this molecule, it protects brain cells from cell death during brain diseases [87]. The role of glutathione in brain diseases and its potential use for the treatment of brain diseases has been extensively reviewed and described in recent literature [88]. An overview of the receptors that have been discussed here and the influence of diseases on their expression has been given in **Table 1**.

3. Expert opinion

The blood–brain barrier (BBB) maintains the homeostasis of the brain by regulating particularly the influx/efflux and metabolism of endogenous compounds and exogenous drugs. Intracellular systems are available for the degradation of proteins and DNA/RNA. All these processes protect the brain but are unfavorable in targeting macromolecular drugs across (or to) the BBB. In recent papers, the use of receptor-mediated transcytosis to target the brain has been described (see paragraph 1.2). Although these studies show successful delivery, the efficiency is often low and/or methods have limited applicability in humans. In this expert opinion we would like to emphasize the importance of investigating different cellular transport systems when studying drug targeting to the healthy and diseased brain. Moreover, it is our opinion that a differential drug targeting approach should be applied to treat CNS diseases that are related to the BBB specifically and CNS diseases that are related to both the brain and BBB.

To treat the BBB, we propose to apply a protein/RNAi approach where the drug has been coupled to the carrier molecule. However, this approach may have drawbacks since the coupling of a therapeutic protein/siRNA to a carrier molecule may decrease the affinity for the receptor/transporter, but also its biological activity, and therefore its therapeutic efficacy. Moreover, these drugs may also give rise to immuno-reactivity, the formation of antibodies and a faster clearance following subsequent administrations. Therefore, to deliver such a protein or siRNA to the BBB, we propose a Trojan horse liposomal system that is coated with a suitable carrier/targeting molecule. To treat the brain and the BBB we propose to target a plasmid (preferably in a liposomal system) to the BBB and to use the BBB as a protein factory for the production of siRNA or therapeutic proteins that can be excreted.

In this expert opinion we have already mentioned that the integrity of the BBB is changed under disease conditions. This happens particularly under inflammatory disease conditions that occur in many CNS diseases, leading to changed BBB functionality (including permeability; see paragraph 2). This may be due to up/downregulation of transporters, increased paracellular permeability and increased pinocytotic activity. This should be taken into account when studying drug action in the diseased brain. Various approaches that are used to target the diseased brain may fail or be successful because of one or more of the above-mentioned changes in BBB permeability. It is our opinion that the increased endocytotic (pinocytotic) transport (e.g., transcytosis) should not be neglected in the evaluation of CNS effects following the receptor-mediated targeting of proteins and DNA/RNA to the brain. It is possible that the observed effects in receptor targeting studies are both a consequence of the specific targeting molecules, but also of the aforementioned disease conditions related effects on BBB integrity. Such (increased) transport can be very helpful in the therapeutic treatment of CNS diseases and it is in our opinion important to investigate the extent to which these altered states of transport contribute to the delivery of drugs to the brain. Furthermore, it is not unlikely that the ability of carrier molecules to cross the BBB is influenced by the progression of the disease that is being treated. Using the right control groups and studying both plasma- and neuro-pharmacokinetics is essential to accurately study drug transport to the brain in diseased and healthy animals.

Chronic treatment of the brain using targeting strategies to receptors may interfere with the endogenous metabolism of these receptors and may result in an immune response to the carrier/macromolecular drug complex. To avoid this problem it seems more feasible to use such systems in treating acute brain diseases like brain tumors, encephalitis or stroke, in contrast to chronic diseases like Alzheimer's disease, epilepsy and Parkinson's disease. For chronic treatment, gene therapy may be an alternative approach since gene therapy ideally results in long-term expression of a transgene that is beneficial to the treatment of the disease.

Degradation of macromolecular drugs after cellular uptake is dependent on the uptake mechanism and the intracellular pathway of the carrier–drug–receptor complex.

The targeting strategies summarized in this review show receptor-mediated uptake of drugs and the presence of these receptors at the abluminal side of the BBB. The intracellular pathways may involve: escape from the lysosomal and proteasomal degradation system, endosomal escape, followed by diffusion and exocytosis. The exact pathway of degradation of the carrier molecule is for most approaches not completely clear. Moreover, *in vitro* studies performed in our group using DT_R targeting revealed high amounts of drug in the brain capillary endothelial cells, of which a part was able to enter the brain.

New strategies that focus on BBB targeting instead of transport across the BBB may be feasible and very useful. First of all this can be achieved by targeting proteins that have their therapeutic target in the brain endothelial cells. Possible applications are diseases that cause inflammatory conditions in endothelial cells or infectious diseases like encephalitis. An alternative approach is the delivery of genes to the endothelial cells of the BBB. Subsequently the endothelial cells of the BBB will incorporate the delivered gene and start to produce a therapeutic protein. This can be accomplished by several of the presently available technologies involving receptor-mediated uptake or by exploiting the increased pinocytotic transport that is common to the BBB during disease conditions (described above). Such genes may encode for proteins adapted with signaling peptides that target them to specific locations, such as the cell membrane or other intracellular or extracellular targets, depending on the required function and place of action of the encoded protein. This can modify the BBB and make it function like a protein factory that delivers its therapeutic proteins to the brain. This approach can be directly applied to existing (targeting) technologies following i.v. administration. We think that using the BBB as a protein factory can lead to efficient delivery of therapeutic proteins into the brain. At the same time, however, drawbacks such as potentially decreased biological activity following the coupling of a therapeutic protein or siRNA to a signaling peptide and the possibility to initiate an immune reaction may hamper its therapeutic efficacy. These drawbacks should not be neglected when designing a specific treatment approach.

Despite the difficulties in brain targeting, recent publications show not only progress in the well-established methods, but also new methods for receptor-mediated brain targeting. The development of some compounds seems close to clinical application. This can hopefully result in better treatment of well-known severe brain diseases by applying the enormous potential of therapeutic macromolecules that are already available.

Declaration of interest

AG de Boer is shareholder of to-BBB Technologies BV in the Netherlands.

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